Platelet-Rich Plasma Attenuates 30-kDa Fibronectin Fragment-Induced Chemokine and Matrix Metalloproteinase Expression by Meniscocytes and Articular Chondrocytes

Platelet-Rich Plasma Attenuates 30 kDa Fibronectin Fragment Induced Chemokine and MMP Expression by Meniscocytes and Articular Chondrocytes

Abstract

Background: Proteolytic fragments of fibronectin have catabolic effects on cartilage and menisci. Platelet-rich plasma (PRP) is increasingly being used in treatment of a range of joint pathologies but it is unknown whether PRP influences fibronectin fragment (FN-f) pro-catabolic activity.

Hypotheses: The pro-catabolic activity of FN-f on meniscocytes and articular chondrocytes is attenuated by co-treatment with PRP.

Study Design: Controlled laboratory study.

Methods: Human meniscocytes were treated with FN-f (30 kDa) with/without PRP co-incubation and gene expression analyzed by cDNA microarray analysis. Validation of altered expression of known and novel chemokine and protease genes was undertaken by real-time PCR in articular chondrocytes and meniscocytes. Chemokine release was assayed by ELISA and intracellular pathway signaling was evaluated by western immunoblotting.

Results: Microarray analysis and RT-PCR showed increased expression of MMP1,

MMP2, MMP3, MMP9, MMP13, IL-6 and IL-8 (CXCL8), CCL5, CCL20 and CXCL10 chemokines in meniscocytes following treatment with FN-f. Upregulation of these genes was significantly attenuated by PRP. Similar results were seen with articular chondrocytes although no change in MMP2 or MMP9 levels were identified. PRP induced suppression of gene expression was associated with activation of Akt and p44/p42.

Conclusions：30 kDa FN-f induced expression of a range of pro-inflammatory chemokines and MMPs including IL-8, IL-6, CCL20, CCL5, CXCL10, MMP1, MMP3 and MMP13 by both meniscocytes and articular chondrocytes is attenuated by PRP treatment.

Clinical Relevance: These observations provide support for the use and further trials of PRP in management of cartilage and meniscal injuries.

Keywords: platelet-rich plasma; fibronectin fragment; chondrocyte; chemokine; matrix metalloproteinase

What is known about the subject:

Proteolytic fragments of fibronectin are released from articular cartilage following impact injury and are increased in synovial fluid of patients with osteoarthritis (OA). These fibronectin fragments (FN-f) have catabolic activity inducing expression of a range of inflammatory mediators and proteases that contribute to both cartilage and meniscus degeneration. Removal of FN-f from OA synovial fluid decreases detrimental indicating that targeting these molecules may be of benefit in attenuating the development or progression of cartilage and meniscal pathology. There is increasing interest and application of platelet-rich plasma (PRP) for the treatment of osteoarthritis and a range of other joint and musculoskeletal conditions. PRP is a key source of molecules involved in tissue repair and regeneration and can deliver a variety of bioactive molecules that have the potential to suppress pro-inflammatory and proteolytic pathways. It is however not known whether PRP acts to inhibit the broad range of recognized and novel inflammatory and proteolytic pathways activated by FN-f in human meniscocytes and articular chondrocytes.

What this study adds to existing knowledge:

In addition to confirming recent observations that FN-f increase expression of a range of matrix metallproteinases and chemokines in meniscocytes and articular chondrocytes we have, for the first time, identified that FN-f also increase expression of MMP9 and CCL5, CCL20 and CXCL10 chemokines in human meniscocytes. Co-stimulation of both meniscocytes and articular chondrocytes with PRP significantly attenuated the FN-f increased expression of chemokines and MMPs providing mechanistic support for the use of intra-articular PRP injection for the treatment of degenerative and traumatic joint conditions including OA.

Introduction

Fibronectin (FN) is a multidomain glycoprotein present in most extracellular matrices (ECM), including cartilage[8] and synovium[27], as a dimeric glycoprotein. The dimeric glycoprotein is formed through a pair of anti-parallel disulfide bonds at the C terminus linking single glycoproteins with a molecular weight of 230–270 kDa.[40] FN isoforms in adult cartilage are significantly different from fibronectins in other tissues and include predominantly the cartilage specific (V + C) isoform (50-80%) with smaller amounts of the of ED-B (+) isoform.[9] Intact FN has important roles in matrix assembly, morphogenesis, cell migration and inflammation through the binding of multiple domains to itself and a range of matrix proteins and cell surface receptors including collagen, fibulin-1, syndecan and integrins.[35] $\alpha5\beta1$ integrin is the major FN receptor expressed by articular chondrocytes and has important roles in regulating chondrocyte responses to mechanical loading.[33, 45, 58]

FN levels are elevated in cartilage in osteoarthritis (OA) as a result of increased production and retention.[59] This increase of FN in OA cartilage is associated with an

increase in FN levels in synovial fluid.[48] As well as being increased in amount, the FN in OA cartilage and synovium is fragmented, comprising FN-fragments (FN-f) of 30–200 kDa.[22] Unlike intact FN these FN-f have catabolic activity.[21] FN-f bind to and penetrate cartilage tissue resulting in proteinase expression and cartilage damage.[60] FN-f show potent catabolic activity increasing expression of Toll-like receptors and inflammatory cytokines including TNF-α, IL-1β, and IL-1α, elevating production of matrix metalloproteinase(MMP) proteases, and suppressing proteoglycan synthesis.[13, 19, 20, 49, 51] FN-f are also released from articular cartilage following impact injury[7], and have been shown to increase production of MMPs by normal and OA meniscocytes in vitro[50], indicating that a general increase in levels of these biologically active fragments in synovial fluid in OA or other joint pathologies is also detrimental to meniscus structure and function.

Removal of FN-f from OA synovial fluid has been shown to diminish detrimental effects on cartilage[22] indicating that targeting these molecules may be of benefit in attenuating the development or progression of cartilage and meniscal pathology. A

specific pharmacological agent that inhibits the catabolic effects of FN-f has not yet been developed but there is increasing interest in the use of biological therapies such as hyaluronan (HA) and platelet-rich plasma (PRP) for the treatment of osteoarthritis and a range of other joint and musculoskeletal conditions.[15, 28, 29, 31, 38]

PRP is a key source of molecules involved in tissue repair and regeneration and can deliver a variety of bioactive molecules. These include a number of growth factors recognized to be important in regulation of chondrocyte proliferation and anabolic function including platelet-derived growth factor, transforming growth factor beta, fibroblast growth factor and insulin-like growth factor 1.[5, 56] Delivery of a cocktail of agents, as is present in PRP, would be expected to be beneficial for repair of cartilage and meniscus injury and enhance tissue repair by stimulation of anabolic activity whilst proinflammatory and catabolic pathways would be inhibited.[2, 26, 53]

The aim of this study was to assess whether PRP has inhibitory effects on expression of pro-inflammatory and proteolytic molecules induced by FN-f in human meniscocytes and articular chondrocytes.

Materials and Methods

Human articular chondrocyte and meniscocyte isolation and culture

Human cartilage and meniscus samples were obtained from surgical discard tissue, with consent (TMU-JIRB No.201305003), at knee joint arthroplasty from patients with OA (n = 43, mean age 72.86 years, range 56-84 years). Residual OA cartilage with predominantly grade II and III lesions (Collins/McElligot system) from each joint was removed and pooled. Cartilage and meniscus tissue were cut into small fragments, incubated with antimicrobial solution, containing 500 IU/mL penicillin (Gibco, Invitrogen, Burlington, Ontario, Canada), 500 mg/mL streptomycin (Gibco) and 2.5 μg/mL Fungizone (Sigma, St Louis, MO, USA) for 4 h, and then washed with sterile phosphate-buffered saline (PBS) before digestion. Cells were extracted by sequential enzymatic digestion with 0.25% trypsin (Gibco) and collagenase type H (Sigma). Extracted cells were re-suspended in 10 mL Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 HAM medium (Gibco) supplemented with 10% FBS (Gibco), 100 I.U./mL penicillin and 100 mg/mL streptomycin; seeded in complete

medium at a density of 5 x 10^5 cell/mL in 60 mm Petri dishes (TPP, Trasadingen, Switzerland); and cultured in a humidified 5% CO_2 incubator at 37°C for further experimental procedures. Cells between passages 3 and 5 were used.

Experimental protocol

Human articular chondrocytes and meniscocytes were seeded at 5 × 10^5 cells/dish and grown as a monolayer for 5 days in 60 mm tissue culture Petri dishes. Cells were washed with sterile PBS twice, placed in serum-free media for 2 hours, and then co-incubated with fibronectin proteolytic fragments 30 kDa at a concentration of 0.5 μg/mL for 24 hours. Freeze-dried powder of PRP was prepared by Regen Lab and Regenkit (Regen Lab, Lausanne, Switzerland) by centrifugation of peripheral blood, using a thixotropic gel for cell separation and citrate as anticoagulant. The obtained PRP was approximately 3.3-fold platelet increase above baseline. Each ampoule of PRP was dissolved with 1 mL of distilled water. The concentrations of two major growth factors were - transforming growth factor-beta1 (TGF- β1) 125.9 pg/mL and platelet-derived growth factor (PDGF) 40.8 pg/mL. 80 μL of PRP solution was added

to cells with 2 mL medium to yield a final concentration of TGF- β1 5.036 pg/mL and PDGF 1.632 pg/mL.

Extraction of RNA and Real-Time Polymerase Chain Reaction

Total RNA was extracted using TRIzol® RNA Isolation Reagents (Invitrogen, NY, USA). For first-strand cDNA synthesis, 2 μg total RNA was used in a single-round reverse-transcription reaction by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qPCR reactions were carried out in a final volume of 20 μL containing 1 μL of 20X TaqMan® Gene Expression Assay probe (Applied Biosystems), 10 μL of 2X TaqMan® Gene Expression Master Mix, 5 μL of RNase-free water, and 4 μL of cDNA. Complement DNAs were amplified with the following condition: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, using a ViiA7 real-time PCR system (Applied Biosystems). Resultant cycle threshold (Ct) values were normalized to the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and analyzed using the

$2^{-\Delta\Delta Ct}$ method. The Taqman Probes used for gene expression studies are listed in Table 1.

Microarray assay and data analysis

RNA was extracted from control and cells incubated with 30 kDa FN-f`. 0.2 μg of total RNA was amplified by a Low Input Quick-Amp Labeling kit (Agilent Technologies, Palo Alto, CA, USA) and labeled with Cy3 (Agilent Technologies). 0.6 μg of Cy3-labled cRNA was fragmented to an average size of 50-100 nucleotides by incubation with fragmentation buffer at 60°C for 30 minutes. Correspondingly fragmented labeled cRNA is then pooled and hybridized to Agilent SurePrint G3 Human V2 GE 8×60K Microarray (Agilent Technologies) at 65°C for 17 h. After washing and drying by nitrogen gun blowing, microarrays were scanned with an Agilent microarray scanner at 535 nm. Scanned images are analyzed by Feature extraction10.5.1.1 software (Agilent Technologies). The microarray data comply with MIAME (Minimum Information About a Microarray Experiment) guidelines, and the raw data have been deposited in a MIAME-compliant database.

Protein Extraction and Western blotting

Following stimulation cells were immediately washed with ice-cold PBS and protein extracted with standard lysis buffer at 4°C for 15 min. Whole-cell lysates were collected after centrifugation at 13,000 rpm for 10 min. Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gel and following electrophoresis were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked overnight at 4°C with 2% BSA in TBST (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). After washing with TBST, blots were incubated at 4°C overnight with primary antibodies (PathScan® Multiplex Western Cocktail I 1/1000; p44/42 MAPK 1/1000; AKT 1/1000) (all from Cell Signaling Technology, MA, USA) diluted in TBST respectively, washed 6 times before incubation with HRP-labeled secondary antibody 1/5000 (DakoCytomation, Copenhagen, Denmark) for 1 h at room temperature. Membranes were rewashed extensively and antibody binding was visualized with Immobilon™ Western HRP Substrate (Millipore). Immunoblots were scanned by a UVP BioSpectrum AC image system (UVP, Upland, CA, USA) and

quantitated using VisionWork LS software (UVP). Anti-alpha-tubulin (1/5000; Abcam, Cambridge, UK) acted as internal control.

ELISA

Quantification of IL-8 (CXCL8) in supernatants of cultured medium was carried out using Quantikine ELISA kits (R&D Systems, Minneapolis, USA).

Statistical analysis

The values were expressed as fold of band intensity of the target gene or protein to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene or alpha-tubulin. The results are expressed as the mean ± SD. Data are analyzed using SPSS (Statistical Package for Social Sciences) statistical software 18.0; all statistical tests use Student's t-test, and the p value < 0.05 is considered statistically significant.

Results

PRP attenuates FN-f induced chemokine gene expression in meniscocytes and articular chondrocytes

Microarray analysis

To investigate the global gene change in meniscocytes as a result of stimulation with 0.5 μg/mL 30 kDa FN-f an initial microarray assay was carried out. Analysis using the GeneSpring GX software showed that expression of 258 genes was significantly altered. Novel findings were the upregulation of several chemokine genes. The top 5 upregulated genes were IL-8 (CXCL8), CCL20, IL-6, CXCL10, and CCL5, respectively (Table 2). To assess whether PRP had any effect on the 30 kDa FN-f induced changes in gene expression meniscocytes were co-incubated with PRP and 30 kDa FN-f. With co-incubation there was a significant downregulation of expression of each of these top 5 upregulated genes rather than the increased expression seen with FN-f treatment alone (Table 2).

Quantitative gene expression

To confirm these results and to establish whether similar effects were seen in articular chondrocytes experiments were undertaken in which changes in gene expression were assessed by Q-PCR. Following 24 hours incubation with 30 kDa FN-f expression of IL-8 (410.1 ± 310.9 fold), CCL20 (287.8 ± 50.3 fold), IL-6 (281.0 ± 199.4 fold), CXCL10 (859.9 ± 665.2 fold), and CCL5 (167.7 ± 90.4 fold) was significantly increased in meniscocytes. These changes were attenuated by co-treatment with PRP for each of the 5 genes: IL-8 (18.1 ± 13.7 fold, $p = 0.042$); CCL20 (14.5 ± 11.5 fold, $p = 0.0008$); IL-6 (12.7 ± 7.8 fold, $p = 0.0362$); CXCL10 (6.2 ± 5.6 fold, $p = 0.0448$); CCL5 (2.9 ± 2.1 fold, $p = 0.011$) (Figure 1A). 30 kDa FN-f also significantly upregulated expression of IL-8, CCL20, IL-6, CXCL10, and CCL5 in articular chondrocytes (164.4 ± 78.2 fold, $p = 0.0095$; 122.6 ± 76.34 fold, $p = 0.0014$; 43.8 ± 22.9 fold, $p = 0.033$; 186.7 ± 140.4 fold, $p = 0.042$; 14.29 ± 11.27 fold, $p = 0.003$, respectively). These changes were significantly attenuated by co-treatment with PRP (IL-8 - 25.3 ± 22.1 fold, $p = 0.005$; CCL20 - 18.7 ± 9.5 fold, $p = 0.0009$; IL-6 - 10.7 ± 7.9 fold, $p = 0.034$; CXCL10 - 8.6 ± 4.9 fold, $p = 0.044$, and CCL5 - 2.4± 1.9 fold, $p =$

0.003, respectively) (Figure 1B). With PRP treatment alone there was no significant change in expression of IL-8, CCL20, IL-6, CXCL10, and CCL5 genes in either meniscocytes or articular chondrocytes over the time period tested.

PRP reduces FN-f induced IL-8 secretion by meniscocytes and articular chondrocytes

IL-8 secretion by primary meniscocytes was significantly increased following 30 kDa FN-f stimulation for 24 hours (603.59 ± 594.81 vs 112.8 ± 32.3 pg/mL, p = 0.0037) (Figure 2A). This enhanced production was significantly suppressed by PRP co-incubation (266.38 ± 214.09 pg/mL, p = 0.0087) (Figure 2A). Treatment with PRP alone had no significant effect on basal IL-8 secretion levels (128.37 ± 51.29 vs 112.8 ± 32.3 pg/mL, p = 0.0583). 30 kDa FN-f had similar but more pronounced effects on articular chondrocytes showing a significant increase in IL-8 secretion following stimulation for 24 hours (5672.86 ± 5266.53 vs 155.15 ± 52.37 pg/mL, p = 0.0026). The increased secretion of IL-8 induced by 30 kDa FN-f was significant suppressed by

PRP but remained above basal levels (1049.26 ± 866.66 pg/mL, p = 0.0024) (Figure 2B). There was a small, but non-significant increase in IL-8 levels when articular chondrocytes were incubated with PRP alone (237.14 ± 97.45 vs 155.15 ± 52.37 pg/mL, p = 0.0523).

FN-f induced MMP expression by meniscocytes and articular chondrocytes is inhibited by PRP

Following incubation of meniscocytes with 30 kDa FN-f for 24 hours gene expression of MMP1, MMP2, MMP3, MMP9, and MMP13 was significantly increased (112.7 ± 58.0 fold; 1.9 ± 0.4 fold; 41.7 ± 22.0 fold; 1.7 ± 0.3 fold; 15.6 ± 2.2 fold, respectively). Co-incubation with PRP significantly attenuated the effect of 30 kDa FN-f on MMP1, MMP2, MMP3 and MMP13 gene expression (6.6 ± 4.2 fold; 1.0 ± 0.2 fold; 3.4 ± 2.0 fold; 2.3 ± 1.3 fold, respectively). MMP9 gene expression was also decreased by co-incubation with PRP but the results did not reach statistical significance. There was no change in gene expression of MMP1, MMP2, MMP3, MMP9, and MMP13 in meniscocytes cultured for 24 hours in PRP alone (Figure 3A).

Articular chondrocyte expression of MMP1, MMP3 and MMP13 genes was significantly upregulated by incubation with 30 kDa FN-f (17.6 ± 11.3 fold; 29.2 ± 18.3 folds; 6.3 ± 5.7 fold, respectively). This effect was significantly attenuated by co-treatment with PRP (3.4 ± 1.9 fold; 4.3 ± 3.1 fold; 2.4 ± 0.6 fold, respectively). In contrast to meniscocytes, MMP2 and MMP9 genes expression was not upregulated by 30 kDa FN-f stimulation. MMP1, 2, 3, 9, and 13 showed no significant gene regulation following PRP treatment alone (Figure 3B).

Akt and p44/42 MAP kinase phosphorylation induced by PRP

To investigate potential mechanisms by which PRP may be influencing chemokine and MMP gene expression we looked at the activation of Akt and p44/42 MAP kinase, both molecules being recognized as important regulatory intracellular signaling pathways in chondrocytes and meniscocytes. Following stimulation with PRP Akt phosphorylation was rapidly increased in meniscocytes and maintained for up to 3 hours (16.63 ± 5.28 fold, $p = 0.0096$ at 0.5 h; 10.47 ± 1.94 fold, $p = 0.0023$ at 1 h; 4.74 ± 2.42 fold, $p = 0.0533$ at 3 h; compared to baseline). p44/42 phosphorylation was similarly increased

over the same time course (5.24 ± 1.23 fold, p = 0.0015 at 0.5 h; 3.66 ± 0.95 fold, p = 0.0033 at 1 h; 4.54 ± 2.29 fold, p = 0.0258 at 3 h, compared to baseline). Under identical conditions similar results were seen when articular chondrocytes were incubated with PRP (Figure 4). Phosphorylation of Akt was increased at each time point (0.5 h = 19.01 ± 5.30 fold, p = 0.0016; 1 h = 21.26 ± 4.45 fold, p = 0.0005; 3 h = 12.47 ± 5.91 fold, p = 0.0122, compared to baseline). Phosphorylation of p44/42 MAP kinase followed an analogous pattern (0.5 h = 3.58 ± 2.06 fold, p = 0.0487, 1 h = 3.33 ± 2.62 fold, p = 0.1175, 3 h = 2.67 ± 2.47 fold, p = 0.204, compared to baseline).

Discussion

In this study we aimed to assess the influence of PRP on FN-f induced pro-inflammatory and proteolytic activity of human meniscocytes and articular chondrocytes. Using a gene microarray we have identified that 30-kDa FN-f induces increased gene expression of several chemokines in meniscocytes and chondrocytes. In addition to confirming observations that FN-f increases expression of MMP1, MMP2, MMP3, MMP13, IL-6 and IL-8 in meniscocytes[50] we have, for the first time, identified that FN-f also increases expression of MMP9 and CCL5, CCL20 and CXCL10 chemokines in human meniscocytes. Similar effects are evident in articular chondrocytes although we found no change in MMP2 or MMP9 expression in these cells consistent with findings by others.[49] Importantly, co-stimulation of both meniscocytes and articular chondrocytes with PRP significantly attenuated the FN-f increased expression of chemokines and MMPs.

Currently 44 human chemokine ligands 21 chemokine receptors have been described. Chemokine receptors contain 7 transmembrane domains and are G protein-coupled.[42]

Whilst chemokines act as critical extracellular mediators of cell migration, particularly in the immune system[34] there is increasing interest in potential roles as inflammatory mediators in joint tissues. Chondrocytes and meniscocytes express numerous CC and CXC chemokines, including CCL2 (MCP-1), CCL3 (MIP-1α), CCL11 (eotaxin-1), CXCL1, CXCL2, CXCL3, IL-8 (CXCL8), in addition to a number of chemokine receptors which potentially play important roles in activating catabolic pathways.[23, 47, 50] The pattern of chemokine and chemokine receptor expression in normal and OA chondrocytes suggests that chemokines have an impact in cartilage homeostasis and release of matrix-degrading enzymes in normal cartilage remodeling and cartilage breakdown.[11] Chemokine levels are increased in synovial fluid in patients with osteoarthritis.[18] Chemokine and chemokine receptor expression is also elevated in damaged menisci[6] indicating that chemokine production in cartilage or meniscus due to biomechanical injury is important in the development of degenerative joint disease.[7]

Our observation that meniscocytes, in addition to chondrocytes, express CCL5, CCL20 and CXCL10 chemokines and that expression of these cytokines is increased in

articular chondrocytes and meniscocytes on exposure to the proinflammatory 30 kDa FN-f is novel. The effect of increased expression of these chemokines in cartilage and menisci is not yet clear. Whilst IL-1β and high-mobility group protein 1 (HMGB1) increase expression of CCL5, CCL20 and CXCL10 chemokines in human chondrocytes, expression of CCL5 is decreased by the chondroprotective cytokine IL-4 indicating likely roles as pro-inflammatory mediators in the OA. [1, 3, 39]

Cartilage breakdown results in production of metabolically active breakdown products such as FN-f. It is likely, although not yet confirmed that FN-f can also be produced in menisci following acute or chronic degeneration and have effects on all intra-articular tissues. Meniscocytes express a range of MMPs including MMP1, MMP2, MMP3, MMP8, MMP9 and MMP13.[32] Levels of MMPs in synovial fluid are elevated rapidly in patients with a meniscal injury and remain elevated for at least 20 years post injury.[30] Targeting MMP activity within the joint may prevent long-term damage. Pro-catabolic FN-fs appear to act predominantly through the α5β1 integrin receptor.[57] Thus, it would be possible to attempt to influence FN-f activity through targeting this

receptor. However, as single agent therapies are increasingly found to be ineffective there is interest in more broad based biological approaches to treating joint injury and osteoarthritis, especially in its early phase. Targeting pathways activated by FN-f may be an effective means of inhibiting production of multiple mediators of cartilage destruction.[43]

Autologous PRP injections were first used in 1987 in open heart surgery.[14] As a low cost and minimally invasive way to obtain a natural concentration of autologous growth factors, PRP is being studied in different fields of medicine for its ability to aid tissue regeneration.[12,16] PRP has been shown to enhance the healing of meniscal defects in a rabbit model.[24] Preclinical studies showing that PRP enhances chondrocyte viability, proliferation and matrix production provide mechanistic support for the use of intra-articular PRP injection.[25, 36] The optimal concentration, composition and relative importance of each of the components of PRP for clinical use remain unclear. Platelet concentrations of 2.5-3 fold above baseline are considered to be ideal, with higher concentrations potentially inhibiting tissue healing.[17, 46, 55] Furthermore the “ideal”

platelet concentration may depend on the target parameter (e.g., direct promotion of tissue healing or stem cell recruitment), the tissue being treated (e.g., bone, cartilage, or tendon), and stage of disease or wound healing. Consequently, the "ideal" platelet concentration for various clinical scenarios remains unknown. PRP concentration can vary considerably depending on an individual's blood platelet levels from day to day, diet, general health, medication and exercise. The method of PRP preparation also influences platelet concentration and levels of growth factors. PRP used in treatment of osteoarthritis usually contains between 2-6 fold normal platelet concentrations[41] although the optimal protocol for PRP injection in knee OA has not been defined. PRP, approximately 6.8 fold above baseline, inhibits the inflammatory processes in human osteoarthritic chondrocytes[53] whilst a double-blinded, randomized controlled trial using PRP with a platelet concentration of approximately 3 fold above baseline showed benefit over placebo.[37] Other randomized controlled trials have also shown that PRP injection, although with variable preparation formulae, provides symptomatic relief in early knee OA[37], a significantly better clinical outcome compared with HA treatment

in grade III gonarthrosis[10] and efficacy in management of pain and inflammation in OA.[2, 26] The commercially available PRP preparation we used had a platelet concentration of 3.3 fold and is within the 'therapeutic range'. The preparation contained 125.9 and 40.8 pg/mL of TGF-β1 and PDGF respectively which, at the final concentration used in our experimental model system, are at a level at which they would be expected to be biologically active.[44] . In the current study the levels of Akt and p44/42 MAP kinase phosphorylation rapidly increase in both meniscocytes and articular chondrocytes upon incubation with PRP. This is in line with the published literature on Akt and p44/42 MAP kinase phosphorylation following incubation of chondrocytes with a range of growth factors and cytokines.[4]

We have shown that PRP can attenuate the effects of FN-f on both chondrocyte and meniscocyte production of pro-catabolic chemokines and MMPs. The mechanisms by which these effects are produced are not clear but are likely to be multifactorial as PRP can potentially affect numerous overlapping pathways simultaneously due to the presence of a number of anti-inflammatory cytokines and anabolic growth factors.[2]

Interestingly PRP has been shown to stimulate endogenous HA production and show similar effects to HA in the suppression of inflammatory gene and protein expression in synoviocytes and cartilage.[52] High-molecular-weight (800 kDa) HA is believed to be effective *in vitro* and *in vivo* in blocking the catabolic action of FN-f by preventing entry of the fragments into the cartilage.[54] A recent in vitro study comparing the activity of HA and PRP on the expression of anabolic and catabolic genes and inflammatory mediators from human OA cartilage and synoviocytes indicated that whilst both agents decreased catabolic activity, PRP treatment also caused a significant reduction of MMP13, an increase in HAS-2 expression in synoviocytes and an increase in cartilage synthetic activity compared with HA.[52]

Conclusion

In the current study, 30 kDa FN-f induced production of a range of chemokines and MMPs including IL-8, IL-6, CCL20, CCL5, CXCL10, MMP1, MMP3 and MMP13 by both meniscocytes and articular chondrocytes was attenuated by PRP treatment. These observations suggest the mechanism by which PRP might help osteoarthritis and suggests a rationale for continued limited clinical trials. Variations in the composition of PRP from patient to patient are however recognized and composition may also vary depending on the device and protocols used for preparation, methods and time of storage. Due to these limitations and questions remaining on potential interactions with other biologics or materials the current use of PRP in orthopedics needs to be further established.

References

1. Amin AR, Islam AB. Genomic analysis and differential expression of HMG and S100A family in human arthritis: upregulated expression of chemokines, IL-8 and nitric oxide by HMGB1. *DNA Cell Biol.* 2014;33(8):550-565.

2. Andia I, Maffulli N. Platelet-rich plasma for managing pain and inflammation in osteoarthritis. *Nat Rev Rheumatol.* 2013;9(12):721-730.

3. Assirelli E, Pulsatelli L, Dolzani P, et al. Human osteoarthritic cartilage shows reduced in vivo expression of IL-4, a chondroprotective cytokine that differentially modulates IL-1beta-stimulated production of chemokines and matrix-degrading enzymes in vitro. *PLoS One.* 2014;9(5):e96925.

4. Beier F, Loeser RF. Biology and pathology of Rho GTPase, PI-3 kinase-Akt, and MAP kinase signaling pathways in chondrocytes. *J Cell Biochem.* 2010;110(3):573-580.

5. Borrione P, Gianfrancesco AD, Pereira MT, Pigozzi F. Platelet-rich plasma in muscle healing. *Am J Phys Med Rehabil.* 2010;89(10):854-861.

6. Brophy RH, Rai MF, Zhang Z, Torgomyan A, Sandell LJ. Molecular analysis of age and sex-related gene expression in meniscal tears with and without a concomitant anterior cruciate ligament tear. *J Bone Joint Surg Am.* 2012;94(5):385-393.

7. Buckwalter JA, Anderson DD, Brown TD, Tochigi Y, Martin JA. The Roles of Mechanical Stresses in the Pathogenesis of Osteoarthritis: Implications for Treatment of Joint Injuries. *Cartilage.* 2013;4(4):286-294.

8. Burton-Wurster N, Butler M, Harter S, et al. Presence of fibronectin in articular cartilage in two animal models of osteoarthritis. *J Rheumatol.* 1986;13(1):175-182.

9. Burton-Wurster N, Lust G, Macleod JN. Cartilage fibronectin isoforms: in search of functions for a special population of matrix glycoproteins. *Matrix Biol.* 1997;15(7):441-454.

10. Cerza F, Carni S, Carcangiu A, et al. Comparison between hyaluronic acid and platelet-rich plasma, intra-articular infiltration in the treatment of gonarthrosis. *Am J Sports Med.* 2012;40(12):2822-2827.

11. Cristino S, Piacentini A, Manferdini C, et al. Expression of CXC chemokines and their receptors is modulated during chondrogenic differentiation of human mesenchymal stem cells grown in three-dimensional scaffold: evidence in native cartilage. *Tissue Eng Part A.* 2008;14(1):97-105.

12. Daher RJ, Chahine NO, Greenberg AS, Sgaglione NA, Grande DA. New methods to diagnose and treat cartilage degeneration. *Nat Rev Rheumatol.* 2009;5(11):599-607.

13. Dang Y, Cole AA, Homandberg GA. Comparison of the catabolic effects of fibronectin fragments in human knee and ankle cartilages. *Osteoarthritis Cartilage.* 2003;11(7):538-547.

14. Ferrari M, Zia S, Valbonesi M, et al. A new technique for hemodilution, preparation of autologous platelet-rich plasma and intraoperative blood salvage in cardiac surgery. *Int J Artif Organs.* 1987;10(1):47-50.

15. Firestein GS, Zvaifler NJ. Anticytokine therapy in rheumatoid arthritis. *N Engl J Med.* 1997;337(3):195-197.

16. Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-Rich Plasma: From Basic Science to Clinical Applications. *The American Journal of Sports Medicine.* 2009;37(11):2259-2272.

17. Graziani F, Ivanovski S, Cei S, Ducci F, Tonetti M, Gabriele M. The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts. *Clin Oral Implants Res.* 2006;17(2):212-219.

18. Hampel U, Sesselmann S, Iserovich P, Sel S, Paulsen F, Sack R. Chemokine and cytokine levels in osteoarthritis and rheumatoid arthritis synovial fluid. *J Immunol Methods.* 2013;396(1-2):134-139.

19. Homandberg GA, Hui F. Association of proteoglycan degradation with catabolic cytokine and stromelysin release from cartilage cultured with fibronectin fragments. *Arch Biochem Biophys.* 1996;334(2):325-331.

20. Homandberg GA, Meyers R, Williams JM. Intraarticular injection of fibronectin fragments causes severe depletion of cartilage proteoglycans in vivo. *J Rheumatol.* 1993;20(8):1378-1382.

21. Homandberg GA, Meyers R, Xie DL. Fibronectin fragments cause chondrolysis of bovine articular cartilage slices in culture. *Journal of Biological Chemistry.* 1992;267(6):3597-3604.

22. Homandberg GA, Wen C, Hui F. Cartilage damaging activities of fibronectin fragments derived from cartilage and synovial fluid. *Osteoarthritis Cartilage.* 1998;6(4):231-244.

23. Houard X, Goldring MB, Berenbaum F. Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. *Curr Rheumatol Rep.* 2013;15(11):375.

24. Ishida K, Kuroda R, Miwa M, et al. The regenerative effects of platelet-rich plasma on meniscal cells in vitro and its in vivo application with biodegradable gelatin hydrogel. *Tissue Eng.* 2007;13(5):1103-1112.

25. Kruger JP, Hondke S, Endres M, Pruss A, Siclari A, Kaps C. Human platelet-rich plasma stimulates migration and chondrogenic differentiation of human subchondral progenitor cells. *J Orthop Res.* 2012;30(6):845-852.

26. Laudy AB, Bakker EW, Rekers M, Moen MH. Efficacy of platelet-rich plasma injections in osteoarthritis of the knee: a systematic review and meta-analysis. *Br J Sports Med.* 2015;49(10):657-672.

462 **27.** **Lavietes BB, Carsons S, Diamond HS, Laskin RS. Synthesis, secretion, and**
463 **deposition of fibronectin in cultured human synovium. *Arthritis Rheum.***
464 **1985;28(9):1016-1026.**

465 **28.** **Lee AY, Korner H. CCR6 and CCL20: emerging players in the pathogenesis of**
466 **rheumatoid arthritis. *Immunol Cell Biol.* 2014;92(4):354-358.**

467 **29.** **Lisignoli G, Piacentini A, Cristino S, et al. CCL20 chemokine induces both**
468 **osteoblast proliferation and osteoclast differentiation: Increased levels of**
469 **CCL20 are expressed in subchondral bone tissue of rheumatoid arthritis**
470 **patients. *J Cell Physiol.* 2007;210(3):798-806.**

471 **30.** **Lohmander LS, Roos H, Dahlberg L, Hoerrner LA, Lark MW. Temporal patterns**
472 **of stromelysin-1, tissue inhibitor, and proteoglycan fragments in human knee**
473 **joint fluid after injury to the cruciate ligament or meniscus. *J Orthop Res.***
474 **1994;12(1):21-28.**

475 **31.** **Mazzocca AD, McCarthy MBR, Chowaniec DM, et al. The Positive Effects of**
476 **Different Platelet-Rich Plasma Methods on Human Muscle, Bone, and Tendon**
477 **Cells. *The American Journal of Sports Medicine.* 2012;40(8):1742-1749.**

478 **32.** **McNulty AL, Weinberg JB, Guilak F. Inhibition of matrix metalloproteinases**
479 **enhances in vitro repair of the meniscus. *Clin Orthop Relat Res.***
480 **2009;467(6):1557-1567.**

481 **33.** **Millward-Sadler SJ, Wright MO, Lee H-S, et al. Integrin-regulated Secretion of**
482 **Interleukin 4: A Novel Pathway of Mechanotransduction in Human Articular**
483 **Chondrocytes. *The Journal of Cell Biology.* 1999;145(1):183-189.**

484 **34.** **Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte**
485 **trafficking. *Am J Physiol Regul Integr Comp Physiol.* 2002;283(1):R7-28.**

486 **35.** **Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci.* 2002;115(Pt**
487 **20):3861-3863.**

36. Park SI, Lee HR, Kim S, Ahn MW, Do SH. Time-sequential modulation in expression of growth factors from platelet-rich plasma (PRP) on the chondrocyte cultures. *Mol Cell Biochem*. 2012;361(1-2):9-17.

37. Patel S, Dhillon MS, Aggarwal S, Marwaha N, Jain A. Treatment with platelet-rich plasma is more effective than placebo for knee osteoarthritis: a prospective, double-blind, randomized trial. *Am J Sports Med.* 2013;41(2):356-364.

38. Pietrzak WS, Eppley BL. Platelet rich plasma: biology and new technology. *J Craniofac Surg.* 2005;16(6):1043-1054.

39. Platas J, Guillen MI, del Caz MD, Gomar F, Mirabet V, Alcaraz MJ. Conditioned media from adipose-tissue-derived mesenchymal stem cells downregulate degradative mediators induced by interleukin-1beta in osteoarthritic chondrocytes. *Mediators Inflamm*. 2013;2013:357014.

40. Potts JR, Campbell ID. Fibronectin structure and assembly. *Curr Opin Cell Biol.* 1994;6(5):648-655.

41. Pourcho AM, Smith J, Wisniewski SJ, Sellon JL. Intraarticular platelet-rich plasma injection in the treatment of knee osteoarthritis: review and recommendations. *Am J Phys Med Rehabil.* 2014;93(11 Suppl 3):S108-121.

42. Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. *Nat Med.* 1996;2(11):1174-1178.

43. Pulai JI, Chen H, Im H-J, et al. NF-κB Mediates the Stimulation of Cytokine and Chemokine Expression by Human Articular Chondrocytes in Response to Fibronectin Fragments. *The Journal of Immunology.* 2005;174(9):5781-5788.

44. Qureshi HY, Ahmad R, Sylvester J, Zafarullah M. Requirement of phosphatidylinositol 3-kinase/Akt signaling pathway for regulation of tissue inhibitor of metalloproteinases-3 gene expression by TGF-beta in human chondrocytes. *Cell Signal.* 2007;19(8):1643-1651.

45. Salter DM, Hughes DE, Simpson R, Gardner DL. Integrin expression by human articular chondrocytes. *Br J Rheumatol.* 1992;31(4):231-234.

46. Sampson S, Gerhardt M, Mandelbaum B. Platelet rich plasma injection grafts for musculoskeletal injuries: a review. *Curr Rev Musculoskelet Med.* 2008;1(3-4):165-174.

47. Sandell LJ, Xing X, Franz C, Davies S, Chang LW, Patra D. Exuberant expression of chemokine genes by adult human articular chondrocytes in response to IL-1beta. *Osteoarthritis Cartilage.* 2008;16(12):1560-1571.

48. Scott DL, Wainwright AC, Walton KW, Williamson N. Significance of fibronectin in rheumatoid arthritis and osteoarthrosis. *Annals of the Rheumatic Diseases.* 1981;40(2):142-153.

49. Stanton H, Ung L, Fosang AJ. The 45 kDa collagen-binding fragment of fibronectin induces matrix metalloproteinase-13 synthesis by chondrocytes and aggrecan degradation by aggrecanases. *Biochem J.* 2002;364(Pt 1):181-190.

50. Stone AV, Loeser RF, Vanderman KS, Long DL, Clark SC, Ferguson CM. Pro-inflammatory stimulation of meniscus cells increases production of matrix metalloproteinases and additional catabolic factors involved in osteoarthritis pathogenesis. *Osteoarthritis Cartilage.* 2014;22(2):264-274.

51. Su SL, Tsai CD, Lee CH, Salter DM, Lee HS. Expression and regulation of Toll-like receptor 2 by IL-1beta and fibronectin fragments in human articular chondrocytes. *Osteoarthritis Cartilage.* 2005;13(10):879-886.

52. Sundman EA, Cole BJ, Karas V, et al. The Anti-inflammatory and Matrix Restorative Mechanisms of Platelet-Rich Plasma in Osteoarthritis. *The American Journal of Sports Medicine.* 2014;42(1):35-41.

53. van Buul GM, Koevoet WL, Kops N, et al. Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes. *Am J Sports Med.* 2011;39(11):2362-2370.

54. Wang Y, Hall S, Hanna F, et al. Effects of Hylan G-F 20 supplementation on cartilage preservation detected by magnetic resonance imaging in osteoarthritis of the knee: a two-year single-blind clinical trial. *BMC Musculoskelet Disord.* 2011;12:195.

55. Weibrich G, Hansen T, Kleis W, Buch R, Hitzler WE. Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration. *Bone.* 2004;34(4):665-671.

56. Weibrich G, Kleis WK, Hafner G, Hitzler WE. Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. *J Craniomaxillofac Surg.* 2002;30(2):97-102.

57. Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *The Journal of Cell Biology.* 1989;109(2):877-889.

58. Wright MO, Nishida K, Bavington C, et al. Hyperpolarisation of cultured human chondrocytes following cyclical pressure-induced strain: evidence of a role for alpha 5 beta 1 integrin as a chondrocyte mechanoreceptor. *J Orthop Res.* 1997;15(5):742-747.

59. Wurster NB, Lust G. Synthesis of fibronectin in normal and osteoarthritic articular cartilage. *Biochim Biophys Acta.* 1984;800(1):52-58.

60. Xie D, Homandberg GA. Fibronectin fragments bind to and penetrate cartilage tissue resulting in proteinase expression and cartilage damage. *Biochim Biophys Acta.* 1993;1182(2):189-196.

569 **Table 1.** Taqman Probes used for Gene Expression Studies.

GeneSymbol	Accession No.	Applied Biosystems Order No.
IL-6	NM_000600	Hs00174131_m1
IL-8	NM_000584	Hs00174103_m1
CXCL10	NM_001565	Hs00171042_m1
CCL5	NM_002985	Hs00174575_m1
CCL20	NM_004591	Hs00355476_m1
MMP1	NM_002421	Hs00233958_m1
MMP2	NM_004530	Hs01548727_m1
MMP3	NM_002422	Hs00968305_m1
MMP9	NM_004994	Hs00234579_m1
MMP13	NM_002427	Hs00233992_m1
GAPDH	NM_002046	Hs02758991_g1

570

571 **Table 2.** Top of 5 upregulated genes (ranking 1-5) by 30 kDa FN-f stimulation and

572 downregulated chemokine genes by PRP treatment in meniscocytes.

Upregulated genes			Downregulated genes		
Ranking	GeneSymbol	Log Ratio (2)	Ranking	GeneSymbol	Log Ratio (2)
1	IL-8	9.358	1	CXCL10	-7.523
2	CCL20	8.750	4	CCL5	-5.121
3	IL-6	7.563	8	CCL20	-4.464
4	CXCL10	7.412	16	IL-8	-3.951
5	CCL5	6.545	17	IL-6	-3.830

573

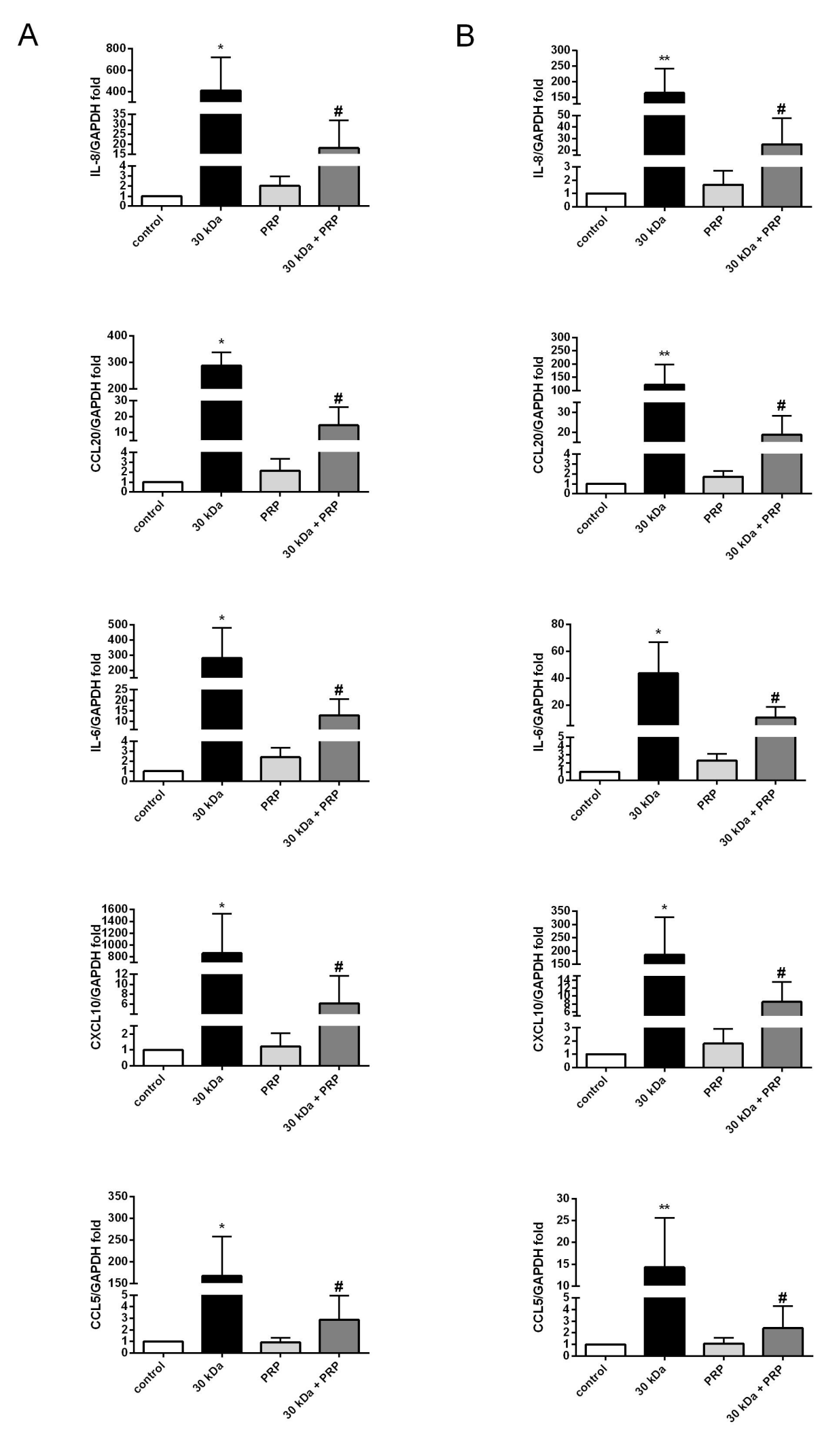
Figure legends

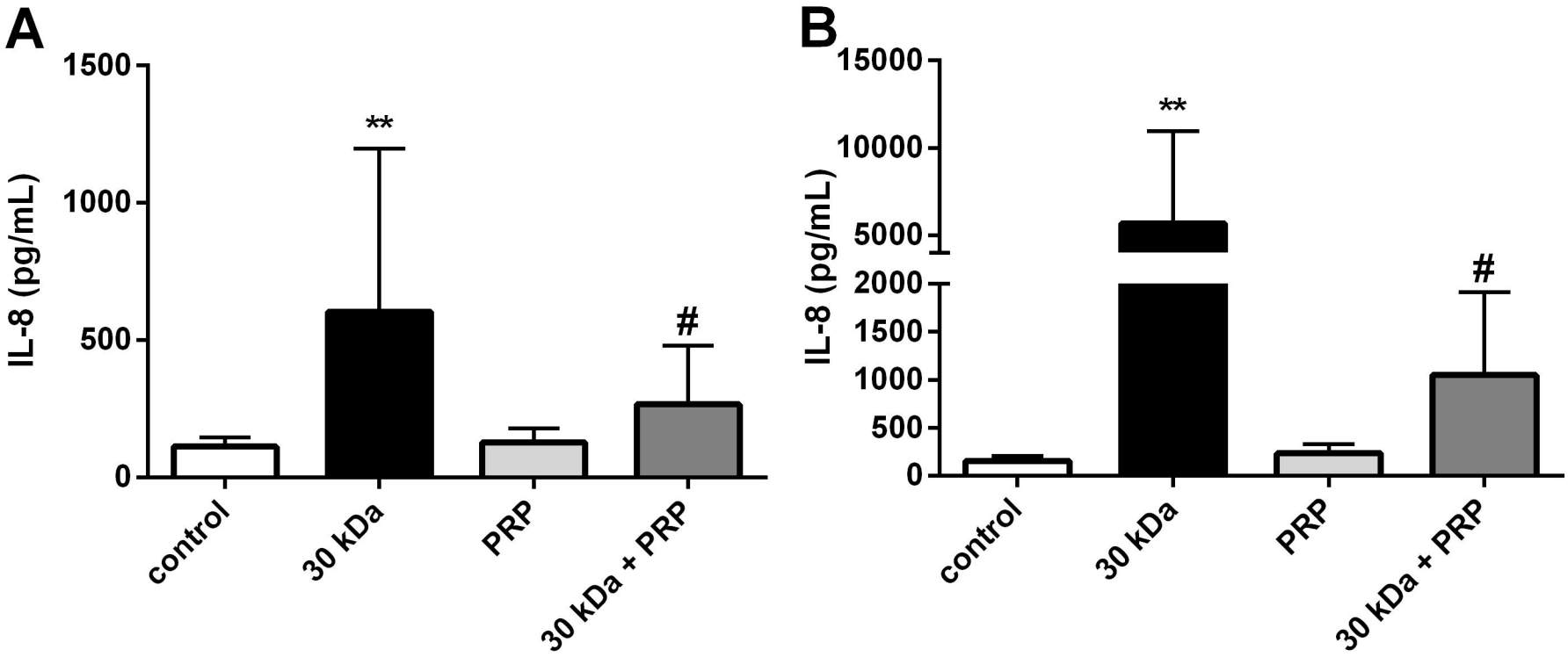
Figure 1. Validation of microarray analysis in both (A) meniscocytes and (B) articular chondrocytes. Top 5 upregulated genes including IL-8, CCL20, IL-6, CXCL10, and CCL5 induced by 30 kDa FN-f all showed significant downregulation by the treatment of PRP. ($^{*}p < 0.05$, compared with control; $^{**}p < 0.01$, compared with control; $^{\#}p<0.05$, 30 kDa+PRP vs 30 kDa).

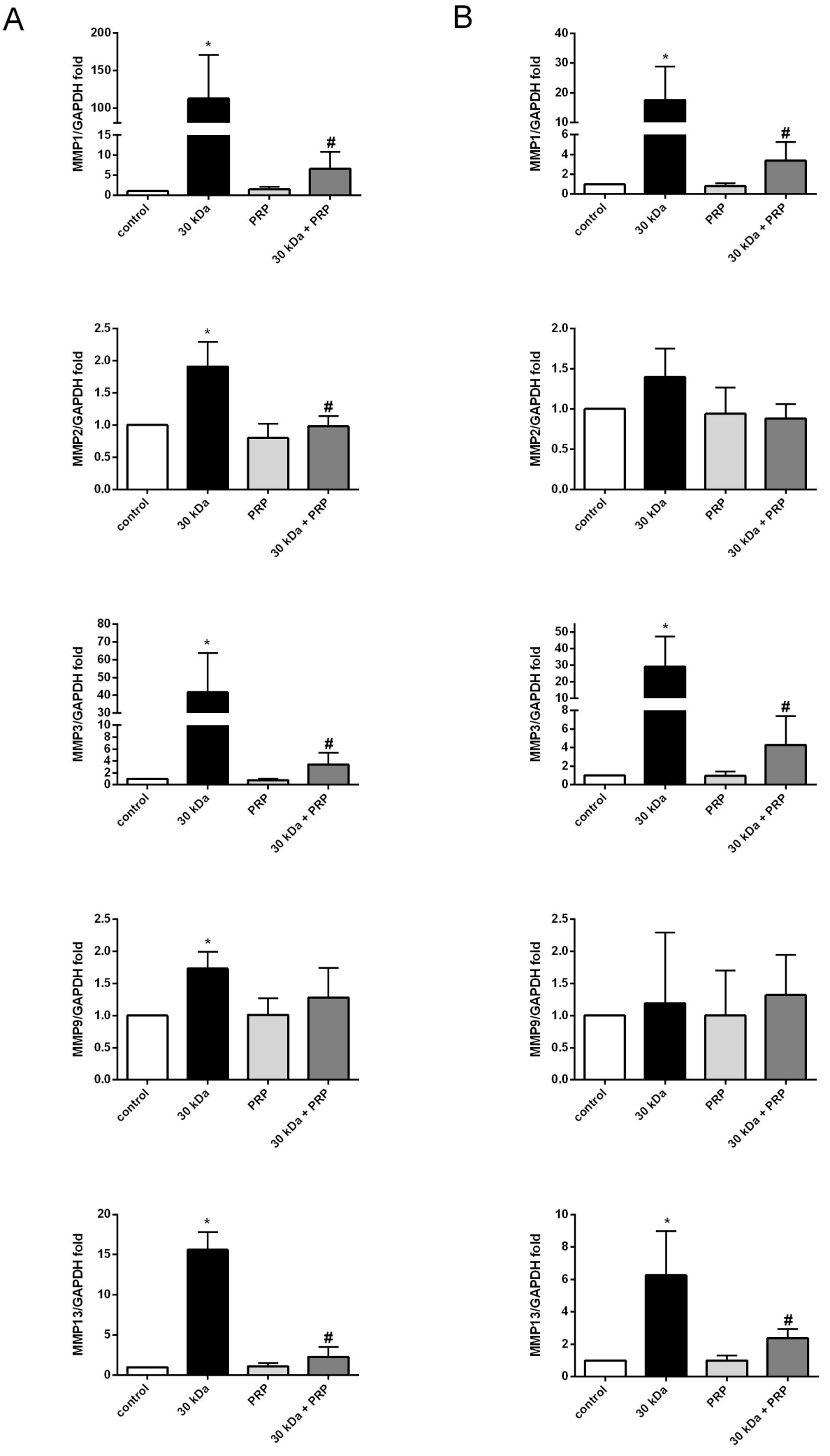
Figure 2. Effects of PRP on 30 kDa FN-f induced IL-8 release. (A) IL-8 release induced by 30 kDa FN-f was suppressed by PRP treatment in meniscocytes. (B) Similar effect was seen in articular chondrocytes. ($^{**}p<0.01$, compared with control; $^{\#}p<0.05$, 30 kDa+PRP vs 30 kDa).

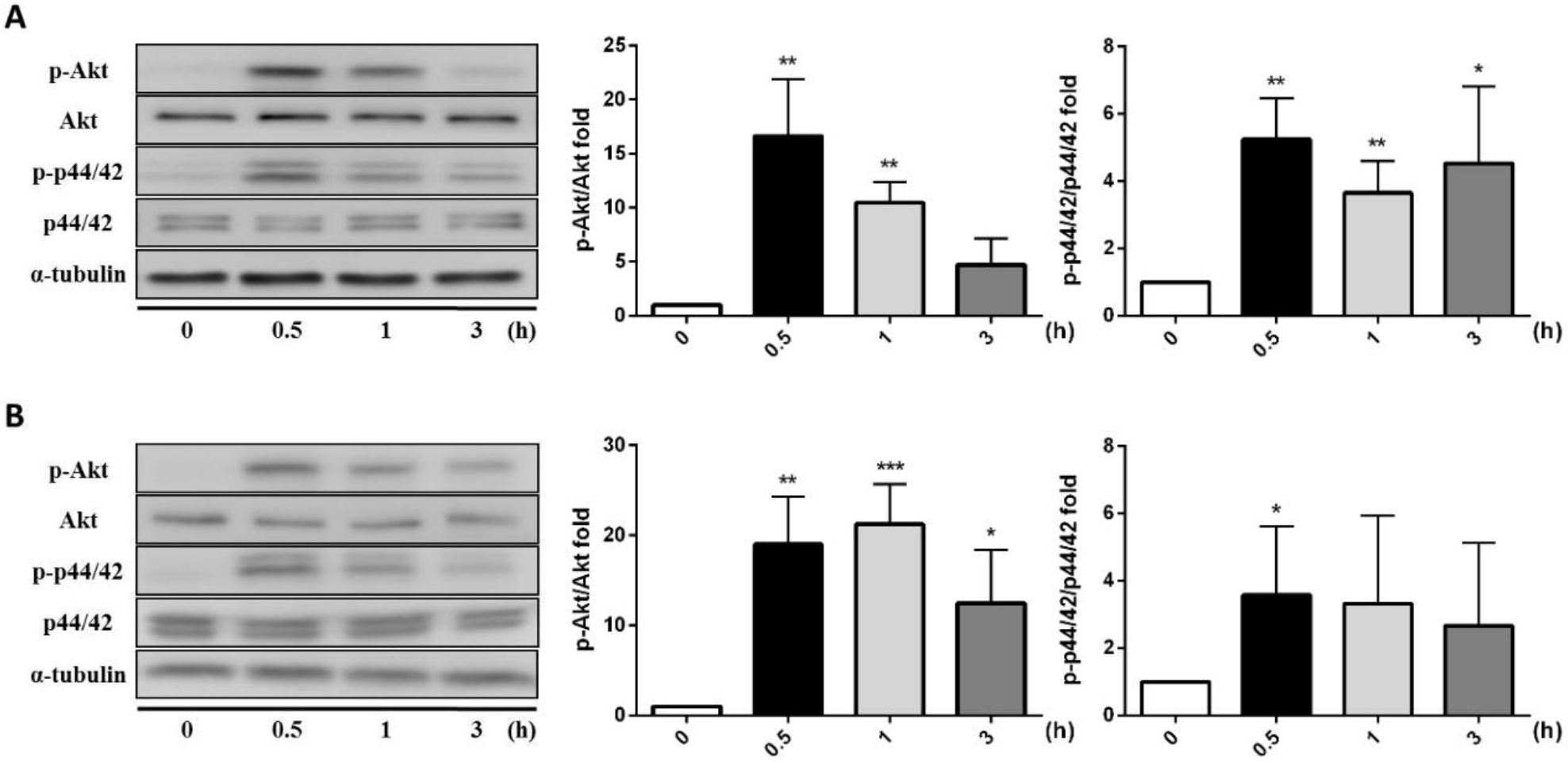
Figure 3. Effects of PRP on 30 kDa FN-f induced MMP expression in both (A) meniscocytes and (B) articular chondrocytes. Upregulation of MMPs induced by 30 kDa FN-f was suppressed by the treatment of PRP in meniscocytes. In articular chondrocytes MMP1, MMP3, and MMP13 gene expression showed the similar pattern. There was no significant change in MMP2 and MMP9 gene expression in articular chondrocytes. ($^{*}p<0.05$, compared with control; $^{\#}p<0.05$, 30 kDa+PRP vs 30 kDa).

Figure 4. Protein phosphorylation of Akt and p44/42 by PRP treatment in human meniscocytes (A) and articular chondrocytes (B). Left panel - representative blots ; right panel - semiquantitative data. Rapid protein phosphorylation of Akt and p44/42 was recognized in both cell types. ($^{*}p<0.05$, compared with control; $^{**}p<0.01$, compared with control; $^{***}p<0.001$, compared with control).


A
B
IL-8/GAPDH fold
CCL20/GAPDH fold
IL-6/GAPDH fold
CXCL10/GAPDH fold
CCL5/GAPDH fold
control
30 kDa
PRP
30 kDa + PRP
*
**
#


A
1500
1000
500
0
IL-8 (pg/mL)
**
#
control
30 kDa
PRP
30 kDa + PRP
B
15000
10000
5000
2000
1500
1000
500
0
IL-8 (pg/mL)
**
#
control
30 kDa
PRP
30 kDa + PRP


A
B
MMP1/GAPDH fold
MMP2/GAPDH fold
MMP3/GAPDH fold
MMP9/GAPDH fold
MMP13/GAPDH fold
control
30 kDa
PRP
30 kDa + PRP
*
#


A
p-Akt
Akt
p-p44/42
p44/42
α-tubulin
0
0.5
1
3
(h)
p-Akt/Akt fold
25
20
15
10
5
0
**
**
0
0.5
1
3
(h)
p-p44/42/p44/42 fold
8
6
4
2
0
**
**
*
0
0.5
1
3
(h)
B
p-Akt
Akt
p-p44/42
p44/42
α-tubulin
0
0.5
1
3
(h)
p-Akt/Akt fold
30
20
10
0
**
***
*
0
0.5
1
3
(h)
p-p44/42/p44/42 fold
8
6
4
2
0
*
0
0.5
1
3
(h)